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Original Article

SACCHARIFICATION OF ALKALINE TREATED RICE STRAW BY SUBSEQUENTLY HYDROLYSIS OF XYLANOLYTIC-CELLULOLYTIC ENZYMES FOR XYLOOLIGOSACCHARIDES AND GLUCOSE PRODUCTION

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ABSTRACT

Rice straw (RS) is a biomass found abundantly in Thailand as a waste product from rice grain collections. This study aimed to produce xylooligosaccharides and glucose from RS. The xylan saccharification of aqueous ammonia treated RS was done by a xylanolytic enzyme cocktail (Xyn10E and GH43B6). The analysis of the final liberated product showed that a majority of xylobiose and minority of xylose and xylooligosaccharides were observed. The cellulose saccharification was subsequently conducted by a cellulolytic enzymes cocktail (Cel9R, Cbh9A, CglT). The synergistic action of the cellulolytic enzyme on alkaline treated and xylanolytic hydrolyzed RS liberated sole glucose as a final product. This is an alternative carrier for the total utilization of RS. The subsequent xylanolytic-cellulolytic enzyme hydrolysis of RS to its subunits leads to a potential feed stock for high value products production.

KEYWORDS: Rice Straw, Saccharification, Xylanolytic Enzyme & Cellulolytic Enzyme

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INTRODUCTION

Background

Biomass refers to an organic matter that is derived from the conversion of sunlight by photosynthesis and stored energy in a chemical bond. The breaking of this chemical bond serves as an energy source for human beings. For instance, lignocellulosic biomass is realized as a sustainable energy sources. Biomass is abundantly found from agricultural residues, herbaceous grasses, and forest products. There are 3 main components build up a recalcitrance structure of plant cell wall, including hemicelluloses, cellulose, and lignin. Cellulose and hemicellulose are macromolecules from different sugars, whereas lignin is an aromatic polymer synthesized from

phenylpropanoid precursors. Cellulose chains are packed into microfibrils via hydrogen bonds. The fibrils are attached with hemicelluloses via hydrogen bonds and linked into a robust network. Hemicelluose is connected to lignin via a covalent bond that results from a recalcitrant structure of the plant cell wall (Shallom and Shoham 2003).

Rice straw (RS) is a biomass derived from rice grain collection that is found abundantly in Asia and South America. RS is a low value material with high bulk density and slow degradation. In Thailand, 37.8 million tons/year of RS are generated (Phitsuwan *et al.* 2016). Conventionally, RS is removed by a burning method, which is the cheapest means available. However, this burning method generates soot, smoke, and air pollution, which is obviously bad for the environmental. Chemically, the composition of RS contains 30% glucan, 10.4% xylan, and 23% lignin (Phitsuwan *et al.* 2016). Hence, RS represents a rich source of fermentable sugar. Due to the bulk of RS collected per year, and that RS is non food material that contains high carbohydrate content, using it as a feed stock for high value substance production is one way to increase its value.

Lignocellulosic biomass refinery research has become an important issue for biomass utilization. A refinery concept was integrated in the production process for the transformation of biomass to a variety of molecular subunits provided for high valued substance production, including biofules, polymers, chemicals, and pharmaceuticals (Kamm *et al.* 2008). The refinery process are includes a pretreatment step for non cellulosic component removal, which is followed by the saccharification of carbohydrate polymer to fermentable sugar. Pretreatment affords the separation of noncellulosic component and the disorganizing of the crystalline structure of carbohydrate polymer, which improves saccharification efficiency. The promising pretreatment process comes with advantages, disadvantages, and economic considerations that have been categorized (Menon and Rao 2012). Hence, the selection of the pretreatment method was based upon several technical and economic factors. The commonly used saccharification methods currently being used are acidic and enzymatic hydrolysis of biomass polysaccharides. Acid saccharification is a strong condition that generates chemical waste, but it is not eco-friendly and less economical because it requires a large consumption of acid in the pilot plant process. Thus, enzymatic saccharification, which is a mild condition process, is an alternative method for polysaccharide saccharification.

Due to the complex structure of biomass, the depolymerlization of biomass requires a group of enzymes to work synergistically to generate the monomer subunits. The xylanolytic enzyme includes non-debranching enzymes and debranching enzymes, play a role in the xylan hydrolysis that results in pentose sugar (Biely 1985). The degradation of cellulose to glucose requires a cooperative action of cellulolytic enzyme endo-1,4-β-glucanase, exo-1,4-β-glucanase, and β-glucosidase (Singhania 2009).

This study thereby presents a method for the utilization of RS. We try to apply subsequent enzymatic hydrolysis to saccharified RS. The fermentable sugar generated from enzymatically saccharification showed the potential to be a feed stock for high value substance production.

METHODS

Feedstock

Rice straw (*Oryza sativa*) was collected from rice fields in the Ayutthaya Province of Thailand. The RS was air dried and cut into small pieces (1-2 cm) before pretreatment.

Pretreatment

Aqueous ammonia was used for RS pretreatment as described by Phitsuwan *et al.* (2016). RS was soaked in 27% (w/w) ammonium hydroxide at a 1:12 ratio (solid:liquid), at room temperature for 14 days. The solid phase was collected and washed with water. RS was neutralized with 1N HCl/NaOH and washed with water. Pretreated RS was dried at 60 ºC and milled with a blender for enzymatic hydrolysis.

Recombinant Enzymes Production

Eschericia coli BL21 (DE3), which harbours a recombinant plasmid, was grown on a Luria Bertani medium and supplemented with antibiotics corresponded with the vector. The culture was incubated at 37 ^{o}C and 200 rpm until the OD_{600} reached 0.6. Protein expression was induced with a 1 mM final concentration of isopropyl β-D-1-thiogalactopyranoside, and then the culture was further incubated at 16 ^{o}C for 16 h. The cells were then harvested and disrupted. Cell-free extracts were applied to His trap^{TM} FF columns (GE healthcare, Little Chalfont, UK) for affinity purification. The purity of purified proteins was determined by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (Laemmli 1970).

Biomass Hydrolysis

Enzymatic hydrolysis of biomass was performed using 1% grinded pretreated rice straw in 50 mM SPB pH 7.0. at 50 °C. An equal µg protein of enzymes were mixed and incubated at 50 °C. Hydrolysis samples were collected at several times and boiled for 15 min to stop reaction. The liberated reducing sugars were quantified by the Nelson-Somogyi method.

Analysis of Hydrolysis Products

Hydrolysis samples were centrifuged at 9,200 x *g* for 1 min and the supernatants were collected. The analysis of the hydrolysis products profile were carried out by TLC as described by Sornyotha *et al.* (2007). The xylose concentration was quantified by a D-xylose assay kit (Megazyme International, Wicklow, Ireland). The glucose concentration was quantified by Glucose liquiColor kit (Human, Wiesbaden, Germany)

RESULTS AND DISCUSSIONS

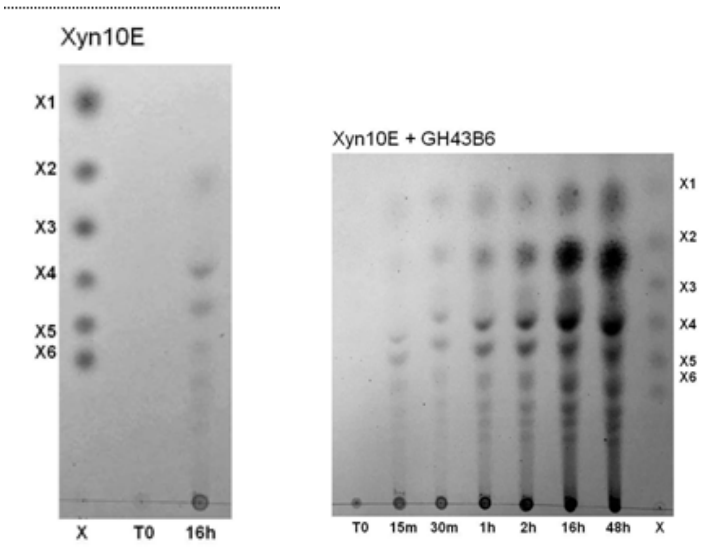
Lignocellulosic biomass is a renewable source of energy. It is derived from plants that convert energy from sunlight and store it in a chemical bond. The major composition of plant cell walls are hemicelluloses, cellulose, and lignin. This study focused on rice straw, as 40% of its carbohydrate content persuaded us to improve technology for RS utilization. The structure of plant cell walls is complicated. Lignin is connected with the carbohydrates in a plant cell wall, forming a lignin shield that blocks the accessibility of carbohydrate degrading enzymes, and which decreases the carbohydrate bioconversion efficiency (Zeng *et al.* 2014). Furthermore, the benzene ring of lignin could form a hydrophobic interaction with the aromatic side chain of the enzyme, causing the absorption of the enzyme on the lignin layer (Berlin *et al.* 2006). Thus, pretreatment is a necessary requirement for improving the digestibility of the enzyme. Pretreatment focuses on the removal or relocalization of lignin (Mansfield *et al.* 1999), increasing the porosity of biomass material (Meng and Ragauskas 2014) and reducing its structural complexity (Van Dyk and Pletschke 2012). In this study, we choose alkali pretreatment using ammonium hydroxide. The aqueous ammonia is an inexpensive reagent and provided abundantly. Moreover, the reagent is reusable, which makes it successful at the pilot scale. Furthermore, aqueous ammonia is less

corrosive to equipment. Using alkali pretreatment results in a high sugar yield with low inhibitor formation (Menon and Rao 2012). It has been reported that the weak base property of aqueous ammonia can selectively remove lignin from various kinds of biomass (i.e; corn stover, barley hull, wheat straw, sugarcane bagasse, plam residue, switchgrass, miscathus, and bamboo) (Phitsuwan *et al.* 2016). Aqueous ammonia cleaves C–O–C bonds in lignin, as well as ether and ester bonds in the lignin–carbohydrate complex, resulting in desirable characteristics that include the swollen structure of biomass (Kim *et al.* 2003). Our laboratory demonstrated that using aqueous ammonia for the pretreatment of rice straw led to a 42.4% delignification, and that afterwards cellulose and xylane content were 39.8 and 11.7%, respectively (Phitsuwan *et al.* 2016). The structural change of biomass showed the disorganization of RS fiber and an increase in porosity, which allowed the enzyme to access to internal surface (Ko *et al.* 2009).

Traditionally lignin is a waste that is obtained from biomass refinery processes and pulp production. The structure of lignin is a polymer of phenylpropenyl and has been established as a chemically synthesis for high value products. (Huang *et al.* 2008). Thus, the extraction and separation of lignin from biomass can be used as a promising aromatic feed stock for synthesis of aromatic compounds. Lignin building blocks showed very high potential for depolymerization to benzene, toluene, and *p*-xylene, which is suitable for the conversion to nylon, resins, and other polymers (Cherubini and Strømman 2011). Additionally, lignin could be depolymerized to oxygen-containing aromatics, which are difficult to produce via petrochemical routes. The aromatic containing products that originated from lignin include coumaric acid hydroxycinnamic acid, ferulic acid, coumaryl alcohol, sinapyl alcohol, and coniferyl alcohol (Cherubini and Strømman 2011).

The carbohydrate content in RS was then saccharified and the Xylan layer that covered cellulose layer was hydrolyzed. In this study we used xylanolytic enzyme hydrolysis for xylan conversion. The GH10 endo-xylanases collection in our laboratory included Xyn10A (Waeonukul *et al.* 2009), Xyn10B (Sudo *et al.* 2010), Xyn10C (Imjongjairak *et al.* 2015), Xyn10D (Sakka *et al.* 2011), and Xyn10E (Sermsathanaswadi *et al.*) was screened for a candidate of RS hydrolysis. GH10 was determined to be effective in reducing sugar liberation from 1% natural RS. The maximum reducing sugar liberation from RS (34.5 µg/mL) was achieved by Xyn10E, after 16 h hydrolysis. Hence, we selected Xyn10E for xylan conversion. Xyn10E was hydrolyzed pretreated RS for 16 h. The hydrolysis products were analyzed on a thin layer chromatography (figure 1 a) and revealed that xylotetraose was a major product. Moreover, it revealed that xylobiose and xylooligosaccharide with a degree of polymerization higher than 4 are minor products. The endo-xylanse Xyn10E showed a random action mode on xylan and resulted in a mixture of xylooligosaccharides. The improvement of hydrolysis efficiency of GH10 is requried the accessory enzyme. A multifunctional GH43 that exhibits exo-β-xylosidase, endo-xylanase, and α-L-arabinofuranosidase (GH43B6) has been cloned and characterized. The exo-β-xylosidase action of GH43B6 hydrolyzed long chain xylooligosaccharide faster than the short chain xylooligosaccharide (Wongratpanya *et al.* 2015). Thus, Xyn10E and GH43B6 were mixed and hydrolyzed with pretreated RS. After 48 h of hydrolysis 1.12 mg/mL of reducing sugar was detected (28% saccharification), which was 4 times higher than a single Xyn10E hydrolysis. The hydrolysis products that were monitored on TLC (figure 1 b) showed that a small amount of xylose was detected at 15 min of hydrolysis and that xylose concentration continuously increased. Furthermore, xylobiose and xylotetraose were found as a major product, and xylopentaose and xylohexaose were also found at 48 h of hydrolysis. The amount of xylose was dramatically higher than single Xyn10E hydrolysis. This result indicated that GH43B6 could hydrolyze the hydrolysis product of Xyn10E and boost the hydrolysis efficiency of Xyn10E.

The liberated hydrolysis products from Xyn10E and GH43B6 could be applied to a vast variety of applications. Xylose is used as feed stock for bioethanol production by genetic modified *Saccharomyces cerevisiase* (Vilela *et al.* 2015). Furthermore, xylose could be converted to furan, which is feed stock for maleic anhydride and furfuryl alcohol production (Kamm *et al.* 2008). Moreover, fungal fermentation of xylose to itaconic acid, which is feed stock for acrylate-base polymer production (Bos *et al.* 2010), is another carrier of the xylose application. Xylose also used as precursor for xylitol, a healthy sweetener, which is produced via microbial fermentation (Winkelhausen and Kuzmanova 1998). Xylobiose could be used as a feed stock for high value chemical production. For instance, Shinoyama *et al.* (1988) demonstrated the biological conversion of xylobiose to alkyl β-xylosides, which is a primer for chondroitin sulphate synthesis. In addition, xylobiose also plays a role as a prebiotic, which enhances the growth of probiotic strains *Bifidobacterium* and *Lactobacillus* sp. (Manisseri and Gudipati 2012). Moreover, some reports have proposed that xylobiose could be fermented to ethanol by recombinant *Klebsiella oxytoca* M5A1 (Burchhardt and Ingram 1992).



(a) **(b)**

Figure 1: Chromatogram of RS Hydrolysis Profile of Xyn10E (A.) and Mix of Xyn10E and GH43B6 (B.) that was Conducted by Thin Layer Chromatograph Hydrolysis Reaction was Incubated at 50°c, Ph 7.0

The major xylan in RS is arabinoxylan, which is highly decorated with the arabinosyl side group (Pitkänen *et al.* 2009). The GH 10 has been reported to attract the substituted region of the xylan backbone, producing oligosaccharide, which contains a substitution side chain (Biely *et al.* 1997). Moreover, GH43B6 has been reported that it is not able to release the arabinosyl side group from long chain xylan and arabinoxylooligosaccharide (Wongratpanya *et al.* 2015). Thus, xylooligosaccharides from Xyn10E and GH43B6 hydrolysis should be contained substituted side group. It has also been reported that Arabinoxylooligosaccharide (AXOS), with a degree of polymerization 3-6, showed prebiotic potential for increasing the community of bifidobacteria in cecum and feces of mice (Santos *et al.* 2006) and humans (Na and Kim 2007). Large AXOS, with a degree of polymerization 15, showed a degree of *Bifidobacterium* growth in chicken's cecum that was higher than fructooligosaccharide (Courtin *et al.* 2008).

The RS hydrolyzed with xylanolytic enzyme was collected and dried. Then RS was further subjected to cellulose saccharification by the cellulolytic enzyme cocktail, which included endo-glucanase Cel9R (Zverlov *et al.* 2005),

cellobiohydrolase Cbh9A (Zverlov *et al.* 1998) from *Clostridium thermocellum*, and β-glucosidase CglT from *Thermoanaerobacter brockii* (Waeonukul *et al.* 2012). The pretreated RS that was hydrolyzed with the xylanlytic enzyme was hydrolyzed with the cellulolytic enzyme cocktail. The 174.9 µg/mL liberated reducing sugar was achieved after 48 h, and the hydrolysis and determination glucose was 21 µg/mL. A comparison with hydrolysis of only alkaline pretreated RS revealed that the xylanolytic enzyme treatment could enhance saccharification of the cellulolytic enzyme, and reducing sugar was increased to 371.04% (figure 2). Moreover, the hydrolysis rate of the cellulolytic enzyme was dramatically boosted up at 30 min of hydrolysis. These results reinforced the fact that removing xylan from RS enhanced cellulose saccharification efficiency. The xylanolytic enzyme breaks the xylan chain that binds to cellulose fibers and allows cellulolytic enzymes direct to cellulose easier. Hydrolysis product profiles of Cel9R were analyzed on TLC showed that the hydrolysis product was limited at cellotetraose (G4) as a major product, and that oligosaccharide G6 and larger were observed at 48h (figure 3a). Cel9R is a processive endo-β-1,4-glucanase, which produces cellotetraose (Zverlov *et al.* 2005). Hence, cellobiohydrolase and β-glucosidase were cooperated with Cel9R to produce glucose. Synergistically working of Cel9R, Cbh9A, and CglT resulted in a sole glucose as the final product (figure 3b). Cellobiohydrolase releases cellobiose from Cel9R hydrolysis product, then, cellobiose is converted to glucose by action of CglT. Moreover, CglT also capable for hydrolyzed G4 to glucose (Waeonukul *et al.* 2012).

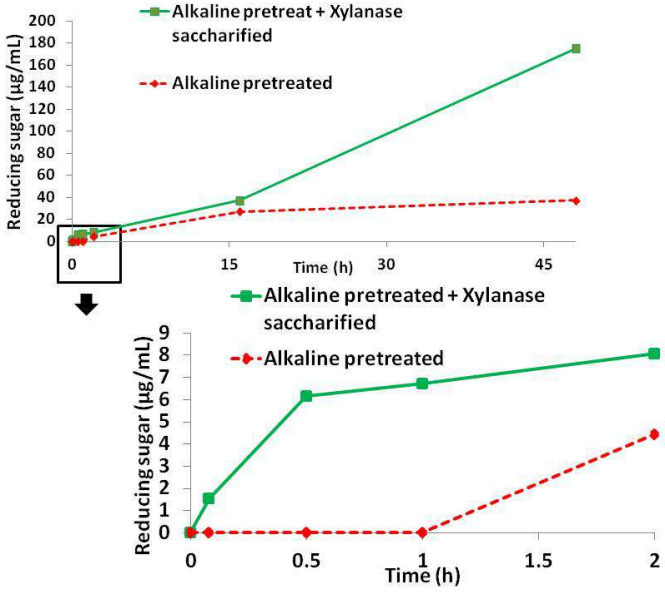


Figure 2: Saccharification of Alkaline Pretreatment RS and Alkali-Xylanolytic Treated RS by Cellulolytic Enzyme Cocktail. Reducing Sugar Was Monitored

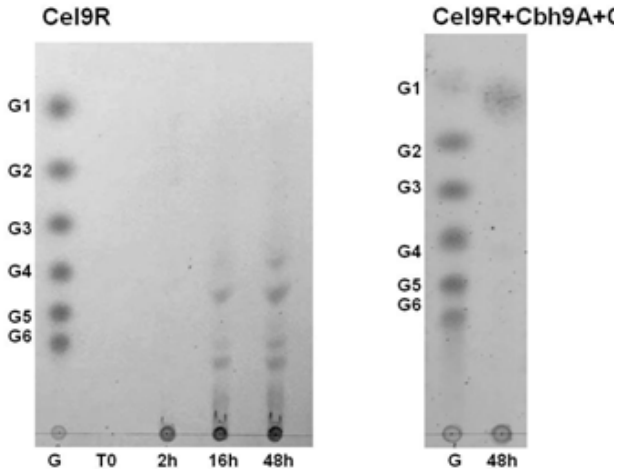


Figure 3: Chromatogram of Alkaline + Xylanolytic Pretreatment RS Hydrolysis Profile by Cel9R (A) and Synergistic Action of Cel9R, Cbh9A and Cglt (B.), Conducted by thin Layer Chromatography. Hydrolysis Reaction was Incubated at 50ºc, Ph 7.0.

Since glucose was fermented by baker's yeast to ethanol, it shows potential as a feed stock for energy production. Furthermore, using glucose as a carbon source for microbial fermentation could be converted to various kinds of high value products (ie; lactic acid and other organic acids). Moreover, glucose could be supplied to the food industry. For instance, the conversion of glucose to sorbitol, which is non calorie sweetener, is applied to functional food. Moreover, the chemically oxidation of glucose to glucaric acid could serve as starting point for nylon and surfactants production (Cherubini and Strømman 2011).

In summary, we demonstrated the process for the utilization of rice straw. Subsequent xylanolytic-cellulolytic enzyme hydrolysis was applied for RS conversion to their components. The hydrolysis product from RS could be used as a feed stock for high value product production. This carrier is an alternative way of increasing the value of RS.

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